Indian Journal of Experimental Biology
Vol.50, August 2012 pp. 517-530

Radiomodulation by Hoechst 33258 against radiation-induced damage in murine splenocytes

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Received 21 November 2011; revised 7 May 2012

In this study modulatory effect of Hoechst 33258 on radiation induced membrane related signaling events which ultimately leads to apoptosis has been investigated. Splenocytes from swiss albino mice were irradiated in air at room temperature in a gamma chamber (240 TBq ^{60}Co Model 4000 A) at the dose-rate of 0.052 Gys^{-1}. Membrane lipid peroxidation, fluidity, specific activities of antioxidant enzymes, levels of nitric oxide, glutathione and apoptosis in presence and absence of different concentrations of Hoechst 33258 has been assayed. DNA binding activity of nuclear factor kappa B and activator protein–1 was also assayed by electrophoretic mobility shift assay. Modulatory effect of Hoechst 33258 was examined at 3 and 5 Gy using different concentrations (10, 20 and 30 μ*M*). Hoechst 33258 was found to inhibit radiation induced peroxidative damage and fluidity and lowered the level of nitric oxide and apoptosis - as evident by DNA ladder assay and FACS, indicating free radicals scavenging potential. Dot plot diagramme clearly showed that 30 μ*M* Hoechst 33258 caused 14% and 19% decrease in apoptotic cells at 3 Gy and 5 Gy of radiation respectively (compared to irradiated control group). Further DNA binding activity of nuclear factor kappa B and activator protein–1 was also inhibited but the antioxidant potential of the cells was enhanced. These findings support that Hoechst 33258 protects the cell from undergoing apoptosis. Hoechst 33258 may have interacted and has an ability to protect splenocytes against radiation induced apoptosis through modulation of membrane-related signaling events and antioxidant status.

Keywords*:* Antioxidant enzymes, FACS, Hoechst 33258, Membrane fluidity, Peroxidative damage, Radioprotector

Cellular damage by ionizing radiation is predominantly mediated through free radicals and resultant reactive oxygen species[1,2]. Interaction of ionizing radiation with water as major cellular constituent results in generation of primary water radical species e.g. e^-_{aq}, $HO^\cdot$, $O_2^{\cdot-}$, H_2, H_2O^+. Direct action of majority of primary radicals to bio-molecules are limited due to their short life time and hence its inability to diffuse up to target molecule. These primary radicals generated during water radiolysis react with molecules like oxygen producing secondary radicals ($HO_2^\cdot$, $RO_2^\cdot$), which are not only relatively stable but may also diffuse to vital cellular targets like DNA, proteins and membrane[3].

Biological membranes play an important role in radiation-induced cell injury and death[4-6]. The membrane organization is an initial step in triggering apoptosis[7]. A correlation was found between unrepaired membrane damage and loss of colony forming ability in cells[8-10], the breakdown of nuclear membrane and chromosomal condensation and damage to cell[11], organization of mitotic spindles, interphase death in non-proliferating cells and disorganization of membrane[12]. Thus the integrity of membrane is essential for normal functions of the cell, and the damage, if not repaired, may lead to cell death. It may be mentioned that radiation-induced alterations in the structure and function of cellular membrane are suggested to serve as a signal to activate component of signal transduction pathways involved in apoptosis[4,6]. Further, radiolytically generated free radicals in plasma membrane and subsequent oxidative stress also suggested to be an important component of signaling process leading to apoptosis. Once triggered, apoptosis proceeds via a cascade of events that is accomplished in few hours independently of triggering agent, e.g., radiation vs drugs[13].

Hoechst 33258 and its ethoxy derivative Hoechst 33342 were shown to have radioprotective ability[14-20], as well as radiosensitizing property[21-23]. Differential

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radiomodifying property of Hoechst 33258 make it a possible tool for radiation therapy of cancer. Radiosensitization is suggested to be mediated by inhibition of topoisomerase 1 and II[22,24,25] and formation of head-to-head adduct (head-to-head *bis*-benzimidazoles) which is toxic to cells[26]. Since tumor cells and tissues are often associated with reduced oxygen tension and elevated levels of topoisomerases, Hoechst 33258 has a higher probability of inducing radiosensitization in tumor cells and tissues. Characteristically poor penetration of Hoechst 33258 through cell layers has potential advantage for preferential protection of normal tissues[27], an efforts are being made to understand the mechanism of protection by this class of compounds. Hoechst 33258 and its derivatives are known to interact and provide the stability to DNA leading to radioprotection both in purified DNA systems, and in cell culture studies[15,16,19,20]. It is quite possible that Hoechst 33258 and its derivatives may also interfere with radiation-induced events involved membrane damage and in turn provide the protection. However, no information is available on the chemical-pathways related to membrane events responsible for its protection.

In the present work, modulation of membrane damage by Hoechst 33258 using splenocytes of Swiss albino mice has been investigated. An attempt has also been made to examine the influence of Hoechst 33258 on apoptosis, DNA binding activity of an oxidative stress responsive transcription factor NF-kB (nuclear factor kappa B), AP1 (activator protein–1), nitric oxide ($NO^{\cdot}$) as well as antioxidant status in irradiated splenocytes. Findings of this study suggested that Hoechst 33258 has an ability to protect biological system against radiation effects through modulations of membrane related events and inhibition of apoptosis.

Materials and Methods

Chemicals—Reduced nicotinamide adenine dinucleotides (NADH), pyrogallol, 1-chloro-2,4-dinitrobenzene (CDNB), RPMI-1640 medium (Roswell Park Memorial Institute medium), FBS (Fetal Bovine Serum) 2,6-dichlorophenolindophenol (DCPIP), reduced glutathione (GSH), 1,6 diphenyl-1,3,5-hexatriene (DPH), thiobarbituric acid (TBA), bovine serum albumin (BSA), 5,5-dithio-bis (2-nitrobenzoic acid (DTNB), N-(1-naphthyl)-thylenediamine (NEDD), sulfanilamide, agarose sodium dodecyl sulphate (SDS), boric acid, acrylamide, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), leupeptin and aproteinin, apoptosis detection kit, Hoechst 33258 (4-(6-(4 methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzo[d]imidazol-2'-yl)phenol (Fig. 1) were from Sigma Chemical Co. (St Louis, MO, USA). NF-kB, AP1 oligonucleotides were obtained from Bangalore Genei Pvt Ltd, (Bangalore, India). All other chemicals used were of analytical grade.

Animals—Swiss albino female mice (6-8 weeks old) maintained in the animal house of the university were used. Standard feed (Hindustan Lever Ltd, Mumbai, India) and water were provided *ad libitum*. Animal experiments were conducted according to the ethical guidelines of the Committee for Control and Supervision of Experiments on Animals, Government of India.

Irradiation of splenocytes with γ—rays-Splenocytes from Swiss albino mice suspended in 1×PBS for enzyme assay and in RPMI-1640 medium for rest of the experiments, were irradiated in air at room temperature in a gamma chamber (240 TBq ^{60}Co Model 4000 A) obtained from the Isotope Division, Bhabha Atomic Research Centre (BARC) (Mumbai, India). The dose-rate used was 0.052 Gys^{-1} and determined by Fricke's dosimetry[28,29].

Preparation of samples—Briefly, animals were killed by cervical dislocation and the spleens removed and washed in PBS. Single cell suspensions were made in RPMI-1640 by crushing the spleen in between frosted slides. 5 µl of it were kept aside for protein determination. Viability of these cells with used concentrations (10, 20, 30 µ*M*) of Hoechst 33258 was determined by trypan blue exclusion method both in the medium and in PBS. Cells were incubated in RPMI-1640 with 1% FBS (v/v) in presence and absence of different concentrations (10, 20, 30 µ*M*) of Hoechst 33258 on ice for 30 min before irradiation[20]. They were used as such for assaying lipid peroxidation (LP), fluidity and AP-1 immediately after irradiation; DNA binding activity of NF-kB and FACS after 30 min post-irradiation. For DNA ladder assay and

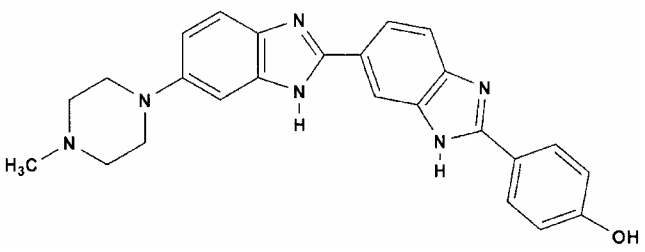

Fig. 1—Chemical structure of Hoechst 33258

$NO^{\cdot}$ determination 5% FBS (v/v) was added in splenocytes (treated with different concentration of Hoechst 33258 in RPMI-1640 without FBS) immediately after irradiation and then post-irradiation incubation was done for 6 h and 24 h respectively. All postirradiation incubation was done at 37 °C in 5% CO_2.

For the assay of antioxidant enzymes, the cells were made and incubated in PBS in presence of different concentrations (10, 20, 30 μ*M*) of Hoechst 33258[20] on ice for 30 min before irradiation. Cells were sonicated at a peak-to-peak amplitude of 18 μ immediately after irradiation and centrifuged thereafter at 16,060 g to remove the debris. Supernatant was collected and used for measurement of specific activities of GST, SOD, catalase and DTD. For GSH content cells were incubated with different concentrations (10, 20 and 30 μ*M*) of Hoechst 33258 in RPMI-1640 without any serum, irradiated and assay was done immediately after irradiation.

Determination of specific activity of superoxide dismutase (SOD)—Superoxide dismutase (SOD) was assayed by the method of Marklund and Marklund[30] by measuring inhibition of pyrogallol at 420 nm. Assay mixtures (1 mL) contained 0.05 *M* sodium phosphate buffer (*p*H 8.0), 0.1 m*M* EDTA and 0.27 m*M* pyrogallol. Reaction was initiated by addition of enzyme sample, which was pretreated with Triton X-100 on ice for 30 min. One unit of enzyme was defined as the amount of SOD required to produce half-maximal inhibition of auto-oxidation of pyrogallol.

Determination of specific activity of DT-diaphorase (DT-D)—DT-diaphorase (DT-D) was assayed according to Ernster *et al.*[31] by measuring reduction of 2,6- dichlorophenolindophenol (DCPIP) at 600 nm with some modifications. The reaction mixture contained 50 m*M* Tris-HCl buffer (*p*H 7.5), 0.5 m*M* NADH, 10 μ*M* DCPIP, 0.08% Triton X-100 and the enzyme sample in a final volume of 1 mL. The reaction was started at 25 °C by addition of NADH and the activity was calculated using an extinction coefficient 21 m*M* /cm. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 μ mole DCPIP/min.

Determination of specific activity glutathione-S-transeferase (GST)—Glutathione-S-transeferase (GST) was assayed using the method of Habig *et al.*[32], by measuring the formation of GSH-CDNB (1-chloro-2, 4-dinitrobenzene) conjugate at 340 nm. Reaction volume (1 mL) contained final concentrations of 0.1 *M* sodium phosphate buffer (*p*H 6.5), 1 m*M* CDNB in ethanol and 1 m*M* GSH. The reaction was initiated by the addition of the enzyme sample. The specific activity was calculated using an extinction coefficient 9.6 m*M*/cm and expressed in terms of μ mole CDNB-GSH conjugate formed min/mg protein.

Determination of specific activity of catalase—Catalase was assayed according to method of Aebi[33], by measuring the decomposition of H_2O_2 (Hydrogen peroxide) to give oxygen and water. The supernatant was treated with Triton X-100 (25 %) for 30 min on ice. Then ethanol (10 μl/ml) was added and again kept for 30 min on ice. The treated supernatant was added to the assay mixture which contained 0.1 *M* sodium phosphate buffer (*p*H 7.0), 10 m*M* H_2O_2 and decrease in absorbance was measured at 240 nm. The activity was calculated using extinction coefficient 0.04 mmole/cm. One unit of catalase activity was defined as amount of enzyme required to decompose one mole of H_2O_2 /min.

Determination of membrane fluidity—A 2 m*M* solution of 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) was prepared in tetrahydrofuran and 100 μL of it was added to 100 mL of rapidly stirring Tris-HCl buffer (10 m*M*, *p*H7.4). Treated and irradiated cells (in RPMI-1640 with 1% FBS) were counted and incubated with 2 m*M* DPH for 30 min at room temperature immediately after irradiation. Fluorescence polarization was measured by excitation with vertically polarized monochromatic light at 365 nm and emission intensity detected at 432 nm through a polarizer oriented either parallel or perpendicular to the direction of the polarized excitation light. The degree of fluorescence polarization was calculated according to Haggerty *et al.*[34]:

$$P = \frac{Ivv - Ivh\,(Ihv / Ihh)}{Ivv + Ivh\,(Ihv / Ihh)}$$

where I is the corrected fluorescence, and v and h indicate values obtained with vertical or horizontal orientation, respectively, of the excitation and analyser polarizer in that order. Fluorescence was monitored on a Shimadzu RF-540 fluorescence spectrophotometer. Because fluidity was inversely related to polarization of the probe, membrane fluidity was expressed as the reciprocal of polarization (*1 / P*).

Estimation of membrane lipid peroxidation (LP)—Membrane lipid peroxidation was estimated

spectrophotometrically by the thiobarbituric acid (TBARS) method as described by Varshney and Kale[35] and was expressed in terms of TBARS formed mg/protein. To avoid interference due to spontaneous peroxidation of membrane and because sufficient amount of TBARS was formed, the assay was performed immediately after irradiation. In brief, 0.5 mL cell suspension (treated and irradiated as explained in section 2.4) was mixed with 1.6 mL Tris KCl (0.15 *M* KCl + 10 m*M* Tris-HCl, *p*H 7.4) buffer to which 0.5 mL 30% TCA was added. Then 0.5 ml, 52 m*M*, TBA (thiobarbituric acid) was added. The tubes were covered with aluminium foil and placed in a water bath for 45 min at 80 ^{o}C, cooled and centrifuged at room temperature for 10 min at 14000 g in a REMI-T8 table-top centrifuge. Absorbance of the clear supernatant was measured against reference blank of distilled water at 531.8 nm in spectrophotometer (UV-160).

Protein determination—Protein concentrations were determined by method of Bradford[36], using bovine serum albumin (BSA) as standard.

Determination of non-protein sulphydryl content—Non-protein sulphydryl content was determined by the method described by Moron *et al.*[37] in splenocytes immediately post-irradiation, using 0.6 m*M* DTNB [5, 5-dithio-bis (2-nitrobenzoic acid]. The absorbance was read at 412 nm and the sulphydryl content calculated with the help of a standard graph made by using different concentrations of reduced glutathione and expressed in terms of μmol/g protein. It was shown that the sulphydryl content determined by this assay using DTNB consisted mainly of reduced glutathione (GSH)[38].

Determination of nitric oxide levels—A total of 10^5 cells were treated as explained in section 2.4 and irradiated in RPMI-1640 (cells were counted in RPMI-1640 and 5% FBS (v/v) was added immediately after irradiation). They were then incubated at 37 ^{o}C in 5% CO_2 for 24 h. This post-irradiation time was chosen as the time point for the assay after careful time course studies because a good amount of $NO^{\cdot}$ was detectable at this time interval (data not shown). After that, cells were pelleted and nitric oxide levels determined in the supernatant by the method of Griess[39] with some modifications. Briefly, to 100 μl of the supernatant, an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalene-ethylene diamine dihydrochloride (NEDD) in 5% orthophosphoric acid) was added and immediately mixed. After 5 min, 200 μl of the above-formed product was transferred to a 96-well flat-bottomed plate and read at 550 nm in a microplate reader. The nitrite content of each sample was evaluated from the standard curve made with sodium nitrite (obtained after linear regression), and was expressed in μ*M*.

Nucleosomal ladder formation assay for apoptosis—Internucleosomal DNA fragmentation was determined by electrophoresis according to the method of Barry and Eastman[40]. Cells were incubated with or without different concentrations of Hoechst 33258 in RPMI for 30 min on ice and irradiated with gamma-radiation (5% FBS (v/v) was added immediately after irradiation). In time-course studies, a clear ladder was observed at 6 h after irradiation compared with earlier time points (data not shown). Therefore, the treated and irradiated cells were then incubated at 37 ^{o}C under 5% CO_2 for 6 h in RPMI. A total of 2% agarose in Tris-borate EDTA buffer was poured into a horizontal gel support. Once the gel solidified, the section of the gel immediately above the comb was removed by cutting along the top side of the comb with a scalpel and filling with 1% agarose, 2% SDS and 64 μg/mL proteinase K. A total of 10^6 cells were centrifuged to remove the medium and resuspended in loading dye and RNAse A (1:1 by volume). It was loaded directly into the wells and electrophoresis carried out at 21 V for 16 h at room temperature. The gel was visualized under illumination after staining with 2 μg/ml ethidium bromide.

FACS analysis—Apoptotic cells were detected based on the principle of Annexin V binding to translocated plasma membrane PS (Phosphatidyl serine). During the apoptotic process, PS translocates from the inner membrane to the outer membrane of the cells[41]. FITC-labeled (Fluorescein isothiocyanate) Annexin-V was added to cultured cells and bound to exposed PS. FITC signals were detected by flow cytometry. PI (Propidium iodide) was added to cultured cells to identify the loss of integrity of the cell membrane, which is specific for necrotic cells. Briefly, treated and irradiated cells in RPMI-1640 with 1% FBS (v/v) (as explained in section 2.4) were incubated for another 30 min in CO_2 incubator at 37 ^{o}C, subsequently washed with PBS, resuspended in 1 × binding buffer (100 m*M* HEPES / NaOH, *p*H 7.5 containing 1.4 *M* NaCl and 25 m*M* $CaCl_2$) at a concentration of approx. 1×10^6 cells/mL. In 500 μL of the cell suspension 5 μL of the Annexin V-FITC and 10 μL of propidium iodide was added, incubated

for 10 min and protected from light. After incubation fluorescence of the cells was determined immediately with a flow cytometer (BDLSR, Becton Dickinson California). The green fluorescence (FITC) and red fluorescence (PI) were detected by filtration through FL-1 and FL-2 band pass filter, respectively. Spectral overlap was electronically compensated using single – color controls including cells alone, FITC alone, and PI alone in separate tubes. Analysis of the multivariate data was performed with Win MDI 2.8 software (Downloaded from internet). $FITC^-$ / PI^-, $FITC^+$ / PI^- or $FITC^+$ / PI^+ represented viable (intact) cells, apoptotic cells, or necrotic cells, respectively (Fig. 5).

Preparation of nuclear extracts—Cells treated and irradiated in RPMI-1640 with 1% FBS (v/v) as explained in section 2.4. Cells were incubated at 37 °C for 30 min only for NF-kB (because maximum binding activity was observed at 30 min post irradiation; data not shown) and used as such immediately after irradiation for AP-1. Subsequently washed twice with PBS, harvested and resuspended in 500 µL buffer containing 20 m*M* Hepes, 1.5 m*M* $MgCl_2$, 10 m*M* NaCl, 0.2 m*M* EDTA, 20% glycerol, 0.1% Triton X-100, 10 µg aprotinin, 4µg leupeptin, 0.2 *M* PMSF and 1 *M* DTT. Incubated on ice for 20 min resuspended in 100 µl of buffer containing 20 m*M* Hepes, 1.5 m*M* $MgCl_2$, 500 m*M* NaCl, 0.2 m*M* EDTA, 20% glycerol, 0.1% Triton X-100, 1µg aprotinin, 0.4 µg leupeptin, 0.02 *M* PMSF and 1m*M* DTT. After constant agitation for 1 h at 4 °C, nuclear debris was pelleted by centrifugation. The supernatant was stored at -80 °C until analysis.

Electrophoretic mobility shift assay (EMSA) for NF-kB and AP-1—EMSA was performed with 4 µg nuclear protein in a total volume of 50 µL in a buffer containing 1 *M* Hepes, 1 *M* $MgCl_2$, 1 *M* NaCl, 100 % glycerol, 1 *M* DTT, 0.5-µg poly [d(I-C)], 40 m*M* EDTA (Ethylenediaminetetra acetic acid) and radiolabelled NF-kB and AP-1 probes at 4 °C for 1 h. The resultant DNA-protein complexes were resolved from free labeled DNA by electrophoresis in non denaturing 8% (w/v) polyacrylamide gel with 0.5 × Tris borate-EDTA electrophoresis buffer[4]. The gels were subsequently dried and autoradiographed.

Statistical analysis—Values are mean of observations made from splenocytes from 18 mice (The experiments were carried out thrice with 6 animals in each group each time). The statistical significance of difference between the data pairs was evaluated by analysis of variance (ANOVA) followed by a Mann-Whitney *U*-test.

Results

Effect on membrane lipid peroxidation—Lipid peroxidation is an important effect of radiation on biological membranes, which brings about various changes in their structure and function[42]. Membrane lipid peroxidation was found to increase with the increase of radiation dose (0-7) Gy[4] (Table 1). To study the modulation 3 and 5 Gy of radiation and different concentrations of Hoechst 33258 were used (10, 20 and 30 µ*M*). Hoechst 33258 decreases radiation-induced (3 and 5 Gy) lipid peroxidation (Table 2). For example, 30 µ*M* Hoechst 33258 caused 37% and 43% decrease as compared to irradiated control group and 20 µ*M* Hoechst 33258 caused 31% and 35% decrease as compared to irradiated control group at 3 Gy and 5 Gy respectively. It was important that in unirradiated control groups, there

Table 1—Effect of different doses of γ-radiation on the levels of lipid peroxidation (LP), nitric oxide (NO^-) fluidity and GSH in splenocytes of Swiss albino mice

[Values are mean + SD of at least 3 experiments]

Treatment	LP (TBARS/mg protein)	NO^- (µ*M*)	Fluidity (1/ polarization)	GSH (µmoles/mg protein)
0 Gy	0.219+0.02 (100)	0.029+0.001 (100)	4.25+0.56 (100)	0.713+0.11 (100)
3 Gy	0.306+0.03** (139)	0.032+0.002 (110)	5.18+0.75** (122)	0.906+0.012** (127)
5 Gy	0.345+0.18** (157)	0.040+0.004*** (136)	5.78+1.10** (136)	0.972+0.04** (136)
7 Gy	0.423+0.14*** (193)	0.045+0.002*** (155)	6.10+0.28* (143)	1.110+0.08*** (156)

Values in parentheses are % change against control. *significantly different (*P<0.001) compared with control (unirradiated with no chemical), **P<0.05 compared with control (unirradiated with no chemical), ***P<0.005 compared with control (unirradiated with no chemical)

seemed to be no adverse effect of Hoechst 33258 on membrane lipid peroxidative damage (Table 2).

Effect on membrane fluidity—Radiation-induced lipid peroxidation has been shown to modulate membrane fluidity. Many membrane-dependent processes are likely to be affected by changes in membrane fluidity[43]. In view of this effect of radiation on fluidity of membrane by using 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) were checked. Immediately after irradiation with different doses (0-7 Gy), the splenocytes were incubated with DPH for 30 min and fluorescence polarization was then measured as described in earlier section (2.9). Polarization is known to be inversely proportional to the fluidity. Like membrane lipid peroxidation, the fluidity of membranes also increased with increase in radiation doses (0-7) Gy (Table 1). The presence of Hoechst 33258 during irradiation decreased the fluidity in a concentration dependent manner (Table 2). Effect of Hoechst 33258 on membrane fluidity is similar as its effect on lipid peroxidation (i.e. fluidity decreased with increase in concentration of Hoechst 33258). Decrease in fluidity of membrane at 30 μ*M* Hoechst 33258 caused 26% decrease at 3 Gy and 28% decrease at 5 Gy of radiation compared to irradiated control group. Hoechst 33258 (20 μ*M*) caused 19% and 23% decrease at 3 Gy and 5 Gy respectively (compared to irradiated control group). It was important that there was not much variation in the effect of different concentration of Hoechst 33258 in unirradiated control group of splenocytes (Table 2).

Effect on levels of nitric oxide—Nitric oxide is endogenously formed in many biological systems and play an important role as signaling molecule and known to be involved in cytotoxic effect. Therefore, in the present study, nitric oxide was determined in terms of nitrite levels. Results showed that nitric oxide levels were elevated progressively in splenocytes following irradiation upto 7 Gy. However, when splenocytes were irradiated in

Table 2—Effect of Hoechst 33258 on the levels of lipid peroxidation (LP), nitric oxide ($NO^{\cdot}$) fluidity and GSH in splenocytes of Swiss albino mice

[Values are mean + SD of at least 3 experiments]

Treatment	LP (TBARS/mg protein)	$NO^{\cdot}$ (μ*M*)	Fluidity (1/ polarization)	GSH (μmoles/mg protein)
0 Gy	0.219+0.02 (100)	0.029+0.001 (100)	4.25+0.56 (100)	0.713+0.11 (100)
+ H (10 μ*M*)	0.214+0.08 (97)	0.028+0.001 (97)	4.17+0.88 (98)	0.681+0.10 (96)
+ H (20 μ*M*)	0.209+0.03 (95)	0.027+0.002 (93)	4.06+1.67 (95)	0.586+0.01 (82)
+ H (30 μ*M*)	0.205+0.02 (93)	0.026+0.01 (90)	3.92+1.50 (92)	0.576+0.07 (81)
3 Gy	0.306+0.03** (139)	0.032+0.002 (110)	5.18+0.75** (122)	0.906+0.012** (127)
+ H (10 μ*M*)	0.238+0.05$^{\#}$ (108)	0.028+0.003 (96)	4.69+0.89 (110)	0.732+0.03$^{\#}$ (103)
+ H (20 μ*M*)	0.210+0.04$^{\#}$ (95)	0.024+0.001$^{**\#}$ (83)	4.19+0.98$^{\#}$ (98)	0.675+0.02$^{\#}$ (95)
+ H (30 μ*M*)	0.192+0.02$^{\#}$ (87)	0.022+0.002$^{**\#}$ (76)	3.91+0.54$^{\#}$ (92)	0.508+0.014$^{**\#}$ (71)
5 Gy	0.345+0.18** (157)	0.040+0.004*** (136)	5.78+1.10** (136)	0.972+0.04** (136)
+ H (10 μ*M*)	0.278+0.06** (126)	0.033+0.001$^{\#}$ (112)	5.07+1.02** (119)	0.784+0.003$^{\#}$ (110)
+ H (20 μ*M*)	0.223+0.05$^{\#}$ (101)	0.028 + 0.001$^{r\#}$ (96)	4.41+1.09$^{\#}$ (103)	0.667+0.02$^{\#}$ (94)
+ H (30 μ*M*)	0.196+0.08$^{\#}$ (89)	0.024+0.002$^{**\#}$ (83)	4. 14+1.32$^{\#}$ (97)	0.521+0.02$^{\#\#**}$ (73)

Values in parentheses are the percentage change against control. *Significantly different ($^{*}P<0.001$) compared with control (unirradiated with no chemical), $^{**}P<0.05$ compared with control (unirradiated with no chemical), $^{***}P<0.005$ compared with control (unirradiated with no chemical), $^{\#\#}$P<0.001 compared with control group (irradiated with no chemical), $^{\#}P<0.05$ compared with control group (irradiated with no chemical). H = Hoechst 33258

presence of different concentration of Hoechst 33258 the level of $NO^{\cdot}$ decreased with increased concentration of this compound (Table 2). For example at 20 and 30 μ*M* of Hoechst the percentage decrease was found to be 25%, 31% at 3 Gy and 30%, 40% at 5 Gy of radiation respectively (compared to irradiated control group). There was not a significant change in the control group due to Hoechst 33258.

Effect on the antioxidant status of the cells—Since antioxidant status is known to determine the radiation response of cells, we have examined the effect of different doses of radiation (0-7 Gy) on enzymes involved in antioxidant function. Splenocytes were irradiated with different doses (0-7 Gy). The specific activities of DTD, SOD, GST and catalase were enhanced at 3 and 5 Gy and then declined at 7 Gy (Table 3). However, the levels of specific activities at 7 Gy are still higher compared to unirradiated controls. Hoechst 33258 was able to inhibit the radiation-induced lipid peroxidation and membrane fluidity. Therefore, it was interesting to see whether Hoechst 33258 could influence the antioxidant status of splenocytes. The splenocytes were incubated with different concentration (10–30 μ*M*) and irradiated at 3 or 5 Gy. The specific activities were determined immediately after irradiation. It was significant that Hoechst 33258 was able to enhance the specific activities of antioxidant enzymes (Table 4).

GSH is an important aqueous–phase antioxidant and an essential co-factor for antioxidant enzymes. GSH plays a crucial role in scavenging the reactive oxygen species/free radicals and in detoxification of drugs[44]. On irradiation of splenocytes with 3, 5 and 7 Gy, levels of GSH were increased (Table 1). When splenocytes were incubated in presence of Hoechst 33258 and irradiated, the levels of GSH were found to be decreased (Table 2). Unexpectedly, Hoechst 33258 decreased radiation induced GSH content when it enhanced levels of other antioxidants, while there was not a significant change in GSH content in unirradiated control group with Hoechst 33258.

Effect on DNA binding activity of NF-kB and AP1—On irradiation of splenocytes, DNA binding activity of NF-kB and AP-1 was found to be increased with radiation dose. Effect of radiation (3 and 5 Gy) and Hoechst 33258 was examined as shown by the complex formation in Fig. 2. However, in presence of Hoechst 33258 the DNA binding activity of NF-kB was decreased (Fig. 2). Hoechst 33258 also decreased the radiation-induced AP-1 band. The free unused oligoprobe is indicated at the bottom of the Fig. 3.

Effect on apoptosis—Biological membranes are considered to be critical target of radiation effect. Recently membrane damage has been found to be a trigger for apoptosis. Lipid peroxidation brings about various changes in the properties of membrane including fluidity and permeability and in turn mediates DNA damage. Since, Hoechst 33258 inhibited radiation-induced lipid peroxidation and membrane fluidity, an attempt has been made to examine effect of Hoechst 33258 on radiation-induced apoptosis in splenocytes using DNA ladder assay. Splenocytes were treated as required, irradiated and subsequently incubated for 6 h before electrophoresis. Presence of Hoechst 33258 during irradiation decreased the extent of apoptosis as found from nucleosomal ladder formation. Fig. 4 shows the effect of Hoechst 33258 on DNA fragmentation at 3 Gy, 5 Gy of radiation and on the unirradiated control group. Above finding were analyzed by the densitometric measurement by using Alpha Imager 3400 programme from Alpha Innotech. It clearly shows that Hoechst 33258 protected the cell against undergoing apoptosis (Fig. 5).

Table 3—Effect of different doses of γ-radiation on the specific activities of antioxidant enzymes in splenocytes of Swiss albino mice

[Values are mean + SD of at least 3 experiments]

Treatment	Specific activity (units/mg of protein) DTD	GST	SOD	CAT
0 Gy	0.006+0.001 (100)	0.021+0.001 (100)	1.366+0.26 (100)	0.855+0.09 (100)
3 Gy	0.009+0.001* (150)	0.026+0.003* (123.8)	1.957+0.07* (143)	1.108+0.1* (129)
5 Gy	0.010+0.003* (166)	0.045+0.003** (214)	2.429+0.2** (177)	1.232+0.17* (144)
7 Gy	0.008+0.003* (133)	0.040+0.012** (191)	2.026+0.13** (148)	1.062+0.07* (124)

Values in parentheses are the percentage change against control. *Significantly different (*P<0.05) compared with control (unirradiated with no chemical), **P<0.001 compared with control (unirradiated with no chemical)

Representive flow cytometry analysis of radiation-induced cell death is given at Fig. 6. Data from dot plot diagrams clearly indicate that radiation-induced apoptosis and to a lesser extent necrotic cell death is decreased in the presence of Hoechst 33258 viz. 30 μ*M* Hoechst 33258 caused 14% and 19% decrease in apoptotic cells at 3 Gy and 5 Gy of radiation respectively (compared to irradiated control group).

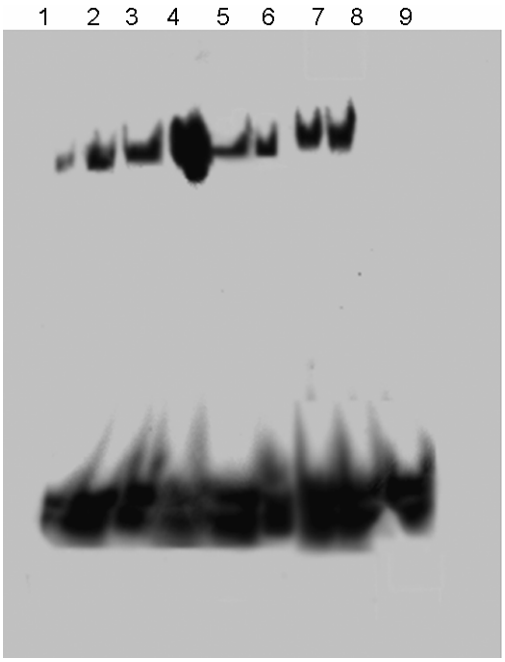

Fig. 2—Effect of Hoechst 33258 on DNA binding activity of NF-kB as assessed by EMSA using nuclear extracts of murine splenocytes treated with Hoechst 33258 on ice for 30 min and subsequently irradiated. Radiolabelled NF-kB oligoprobe was incubated with the nuclear extract. Presence of Hoechst 33258 decreased the NF-kB binding activity as depicted by decrease in complex formation. Free unused oligoprobe is seen at the bottom of the gel. [Lane 1: 5 Gy + 30 μ*M* H; lane 2: 5 Gy + 20 μ*M* H; lane 3: 5 Gy + 10 μ*M* H; lane 4: 5 Gy; lane 5: 3 Gy + 30 μ*M* H; lane 6: 3 Gy + 20 μ*M* H; lane 7: 3 Gy + 10 μ*M* H; lane 8: 3 Gy; lane 9: unirradiated control group; H = Hoechst 33258]

Table 4—Effect of Hoechst 33258 on the specific activities of antioxidant enzymes in splenocytes of Swiss albino mice

[Values are mean + SD of at least 3 experiments]

Treatment	Specific activity (units/mg of protein) DTD	GST	SOD	CAT
0 Gy	0.006+0.001 (100)	0.021+0.001 (100)	1.366+0.26 (100)	0.855+0.09 (100)
+ H (10 μ*M*)	0.007+0.002 (116)	0.022+0.001 (104)	1.608+0.02 (117)	1.006+0.08 (118)
+ H (20 μ*M*)	0.0075+0.003 (125)	0.023+0.002 (109)	1.710+0.74 (125)	1.028+0.5 (120)
+ H (30 μ*M*)	0.008+0.002 (133)	0.025+0.003 (119)	1.820+0.46 (133)	1.069+0.7 (125)
3 Gy	0.009+0.001* (150)	0.026+0.003* (123.8)	1.957+0.07* (143)	1.108+0.1* (129)
+ H (10 μ*M*)	0.010+0.002$^{**\#}$ (166)	0.029+0.005* (138)	2.12+0.11$^{**\#}$ (168)	1.462+0.3$^{**\#}$ (171)
+ H (20 μ*M*)	0.014+0.001$^{***\#\#}$ (233)	0.046+0.001$^{**\#\#}$ (219)	4.167+0.72$^{**\#\#}$ (305)	2.049+0.03$^{**\#\#}$ (239)
+ H (30 μ*M*)	0.017+0.001$^{***\#\#\#}$ (283)	0.051+0.004$^{**\#\#}$ (242.8)	4.414+0.1$^{**\#\#\#}$ (323)	2.241+0.13$^{**\#\#}$ (262)
5 Gy	0.010+0.003* (166)	0.045+0.003** (214)	2.429+0.2** (177)	1.232+0.17* (144)
+ H (10 μ*M*)	0.018+0.001$^{***\#\#}$ (300)	0.059+0.003$^{**\#}$ (280.8)	3.046+0.6$^{**\#}$ (223)	1.601+0.04$^{**\#}$ (187)
+ H (20 μ*M*)	0.019+0.002$^{*\#\#}$ (316)	0.063+0.003$^{**\#\#}$ (300)	4.237+0.5$^{**\#\#}$ (309)	1.687+0.01$^{**\#}$ (197)
+ H (30 μ*M*)	0.021+0.001$^{***\#\#}$ (350)	0.078+0.001$^{**\#\#}$ (371)	5.228+0.23$^{**\#\#\#}$ (382)	1.838+0.03$^{**\#\#}$ (215)

Values in parentheses are the percentage change against control. *Significantly different ($^{*}P<0.05$) compared with control (unirradiated with no chemical), $^{**}P<0.001$ compared with control (unirradiated with no chemical), $^{***}P<0.0001$ compared with control (unirradiated with no chemical). $^{\#}P<0.01$ compared with control group (irradiated with no chemical), $^{\#\#}P<0.001$ compared with control group (irradiated with no chemical), $^{\#\#\#}P<0.0001$ compared with control group (irradiated with no chemical). Abbreviations used are:- H-Hoechst 33258, DTD-DT Diaphorase, GST-Glutathione S- transeferase, SOD-Superoxide dismutase, CAT-Catalase

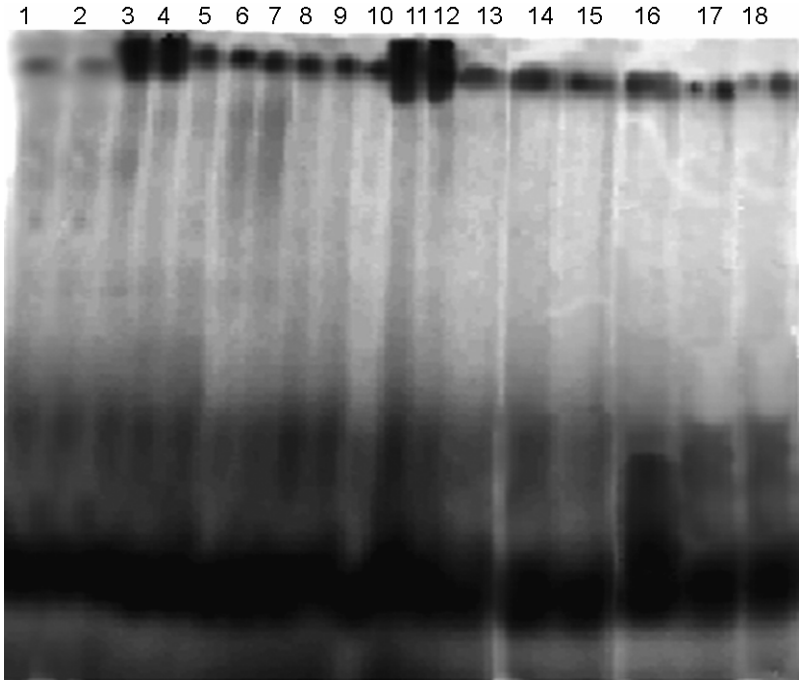

Fig. 3—Effect of Hoechst 33258 on DNA binding activity of AP-1 as assessed by EMSA using nuclear extracts of murine splenocytes treated with Hoechst 33258 on ice for 30 min and subsequently irradiated. Radiolabelled AP-1 oligoprobe was incubated with nuclear extract. Presence of Hoechst 33258 decreased the AP-1 binding activity as depicted by decrease in complex formation. Free unused oligoprobe is seen at the bottom of the gel. [Lane 1-2 (from the left): unirradiated control group; lane 3-4: 3 Gy; lane 5-6: 3 Gy + 10 μ*M* H; lane 7-8: 3 Gy + 20 μ*M* H; lane 9-10: 3 Gy + 30 μ*M* H; lane 11-12: 5 Gy; lane 13-14: 5 Gy + 10 μ*M* H; lane 15-16: 5 Gy + 20 μ*M* H; lane 17-18: 5 Gy + 30 μ*M* H; H = Hoechst 33258]

Discussion

The mechanism of protection of H-258 to DNA is possible because of hydrogen donation, electron or energy transfer from the ligand[45]. The goal of our study was to determine the radioprotective ability of Hoechst 33258 on membrane related signaling pathways, particularly pathways associated with radiation induced apoptosis. Irradiation of mice splenocytes with different doses (0-7 Gy) resulted in a dose-dependent increase in lipid peroxidation[4] (Table 1). The radicals generated from the radiolytic decomposition of cellular water of splenocytes might have reacted with membranes initiating as well as propagating the chain reaction resulting into peroxidative damage. Hydroxyl ($HO^{\cdot}$) radical initiates and the peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals propagate the chain reaction of peroxidation. The removal of $HO^{\cdot}$is expected to inhibit the propagation of radiation-induced lipid peroxidation. It is quite clear from these findings that Hoechst 33258 probably interacted with free radicals and scavenged them and in turn lowered the lipid peroxidation significantly. It is also possible that Hoechst 33258 might have inactivated the free radicals of both the types i.e. which are involved in initiating as well as in propagation of lipid peroxidation process. The conformational transition of the membrane proteins are also linked with lipid peroxidation[46]. In the event of the bindings of Hoechst 33258 with receptor protein complexes of membrane may change the conformation of both acyl chain and cis double bonds in lipids and lead to the inhibition of peroxidation[47]. Such possibility has been suggested in case of other radiomodifiers[48].

Fluidity of splenocyte membrane was increased due to radiation (Table 1). The increase in the membrane fluidity perhaps could be attributed to direct effect on fatty acid chains by phospholipid hydrolysis and therefore their increased mobility[49]. Hoechst 33258 was found to inhibit the radiation-induced change in membrane fluidity of splenocytes (Table 2). It is quite possible that scavenging of free radicals might have resulted in an inhibition of lipid peroxidation and in turn lowered the fluidity of membrane. It may be mentioned that Hoechst 33258 did not affect the membrane fluidity significantly in unirradiated control group of splenocytes. Therefore the protective effect of Hoechst 33258 against the

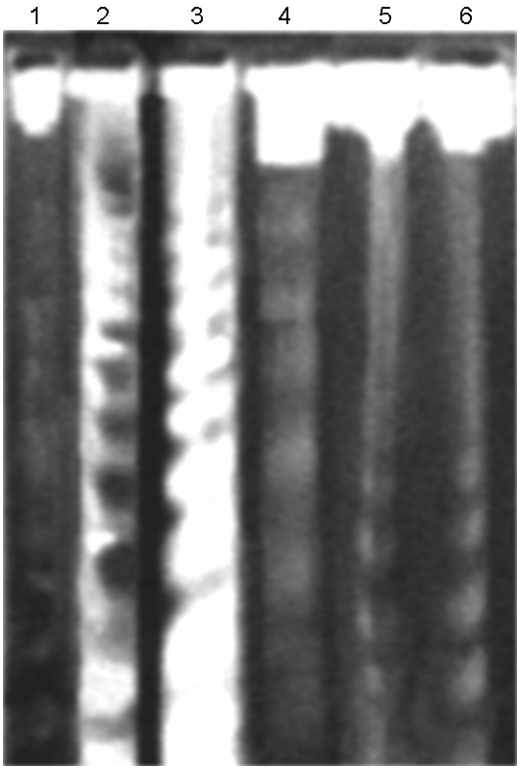

Fig. 4—Effect of Hoechst 33258 on radiation induced apoptosis as assessed by DNA ladder formation. Presence of Hoechst 33258 caused a decrease in apoptotic DNA fragmentation. [Lane 1: 3 Gy + 30 μ*M* H; lane 2: 3 Gy; lane 3: 5 Gy; lane 4: 5 Gy + 30 μ*M* H; lane 5: 0 Gy + 30 μ*M* H; lane 6: 0 Gy; H = Hoechst 33258]

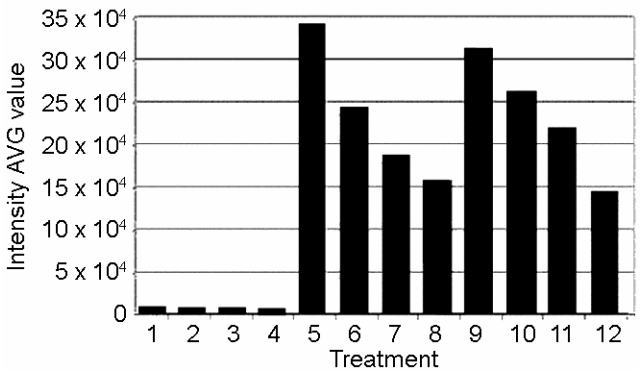

Fig. 5—Densitometric analysis of the apoptotic DNA ladder formation. Preirradiation incubation with Hoechst 33258 causes a decrease in apoptosis. [1; 0 Gy, 2; 0 Gy + 10 μ*M* H, 3; 0 Gy + 20 μ*M* H, 4; 0 Gy + 30 μ*M* H, 5; 5 Gy, 6; 5 Gy + 10 μ*M* H, 7; 5 Gy + 20 μ*M* H, 8; 5 Gy + 30 μ*M* H, 9; 3 Gy, 10; 3 Gy + 10 μ*M* H, 11; 3 Gy + 20 μ*M* H, 12; 3 Gy + 30 μ*M* H. Alpha Imager 3400 of Alpha Innotech, was used for densitometry analysis. AVG is the average value of the density of each pixel detected after background correction (AVG = IDV ÷ area); H = Hoechst 33258].

lipid peroxidation as well as fluidity might be mainly due to its ability to scavenge free radicals. Nitric oxide is known to react rapidly with $O_2^{\cdot-}$ to produce peroxynitrite ($ONOO^-$) which is capable of initiating peroxidative damage[50,51]. Therefore, $NO^{\cdot}$ is likely to contribute to the initiation of radiation–induced lipid peroxidation through reactive species like $ONOO^-$ and $NO_2^{\cdot}$ formed through its interaction with $O_2^{\cdot-}$. As expected, levels of $NO^{\cdot}$ increased in splenocytes on exposure to ionizing radiation (Table 1). These findings also suggested that the concomitant increase of lipid peroxidation and increase in levels of $NO^{\cdot}$ may also be closely interlinked. It is also important that Hoechst 33258 was able to lower the radiation–induced $NO^{\cdot}$ formation in splenocytes. It was quite possible that Hoechst 33258 might have also scavenged $ONOO^-$ and $NO_2^{\cdot}$ radicals generated through interaction of $NO^{\cdot}$ with $O_2^{\cdot-}$.

Modulatory effect of Hoechst 33258 on radiation-induced lipid peroxidation, fluidity change in membranes and generation of $NO^{\cdot}$ clearly support the idea that it has an ability to protect biological systems against radiation effect through interference with membrane related events. Till recently, the work has been focused on binding of Hoechst 33258 with DNA, almost ignoring its role as radioprotector through modulation of membrane related events.

An important component of the signaling process in apoptosis induced by radiation or oxidative stress/ damage suggested to be early generation of free radicals and inturn membrane lipid peroxidation[6,52,53]. Therefore irradiation, lipid peroxidation, membrane alterations and apoptosis appears to be closely linked and our study also supports this. Radiation induced apoptosis in mice splenocytes was also confirmed by flow cytometry analysis. Dot plot diagram showed the possible occurrence of apoptosis in irradiated splenocytes detected by Annexin V–FITC and PI staining. It may be noted that the presence of Hoechst 33258 resulted in decreased levels of radiation-induced apoptosis. For clarity, we have measured the density of each band (Fig. 5). These findings support that Hoechst 33258 protects the cell from undergoing apoptosis. Thus Hoechst 33258 is able to provide the protection to splenocytes against radiation–induced cell death.

Several genes have been shown to be important in apoptosis. The modulation of expression of such genes is mediated by transcription factors. NF-kB and AP-1 are the the transcription factors that has been

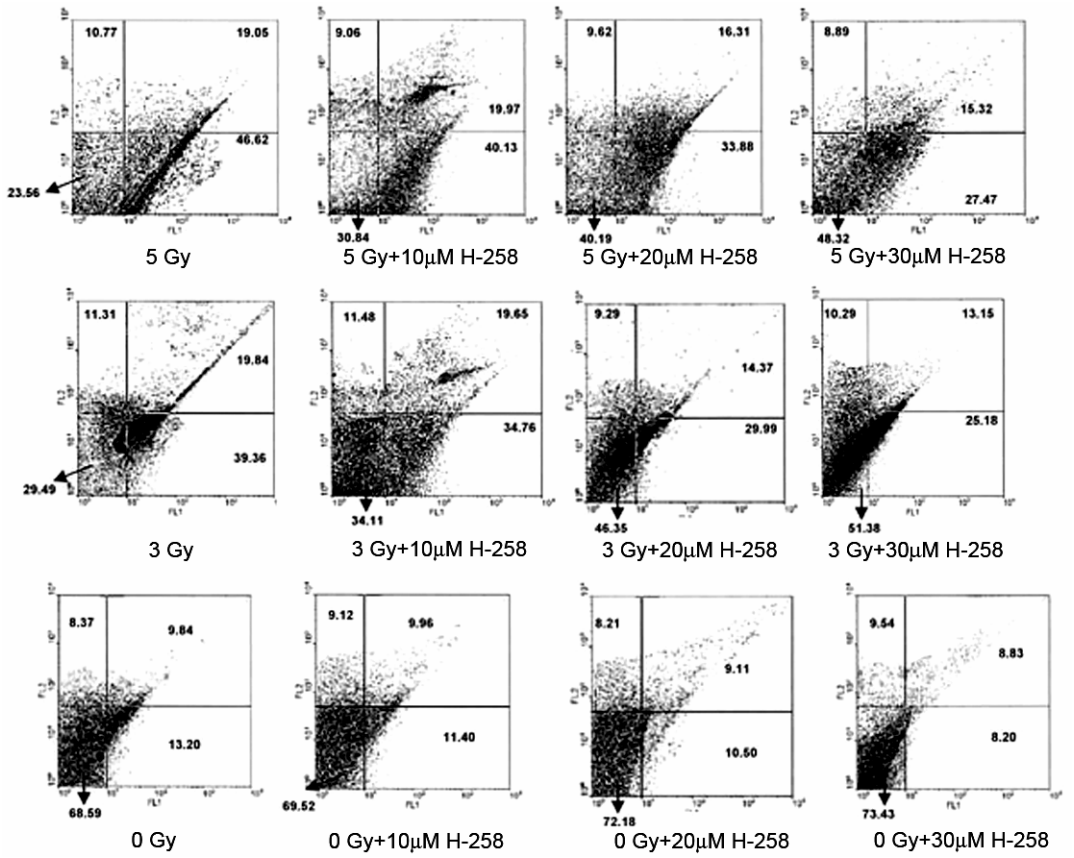

Fig. 6—Dot Plot diagram of irradiated and unirradiated control group (5, 3, 0 Gy) and with 10, 20 and 30 μ*M* Hoechst 33258. This diagram represents typical apoptotic and necrotic cell population detected by Annexin V - FITC and PI staining. The percentages of viable (intact) cells, apoptotic cells, necrotic cells are given in each quadrants of each panel. The lower left quadrant of each panel show the viable (intact) cells which exclude PI and are negative for Annexin V–FITC binding (FITC-/PI-). The upper right quadrants contain the non-viable, necrotic cells, positive for Annexin V –FITC binding and for PI uptake (FITC+/PI+). The lower right quadrants represent the apoptotic cells, positive for Annexin V–FITC and negative for PI (FITC+/PI-). H-258 (Hoechst 33258).

shown to be activated by free radicals[54-58]. NF-kB is stored in the cytoplasm as an inactive complex. The signaling cascade responsible for activating NF-kB and AP-1 is initiated at or near the plasma membrane[59], which is a site for lipid peroxidation. AP-1 is consists of a collection of structurally related transcription factors belonging to the *jun* and *fos* families. Ionizing radiation stimulates the expression of *c-jun* and *c-fos* genes. Thus, the membrane lipid peroxidation, NF-kB, AP-1 and apoptosis might be interlinked. Our studies also showed the involvement of AP-1 in radiation–induced apoptosis as the result of present study showed the increased DNA–binding activity of NF-kB and AP-1 in irradiated splenocytes (Figs 2 and 3). Apart from stimulating the production of P^{53}, the increased DNA binding activity of NF-kB may induce transcription of specific death genes involved in apoptosis. In the presence of Hoechst 33258 during irradiation of splenocytes resulted in decreased DNA binding activity of NF-kB and Ap-1 (Figs 2 and 3). Scavenging of free radicals by Hoechst 33258 might have avoided the initiation of a cascade of signaling pathways which leads to apoptotic cell death, which is evident from the decreased DNA binding activity of NF-kB and AP-1.

Survival of radiolytically damaged cells could probably also depend on their ability to maintain optimal function. The enzymes involved in the metabolism of reactive oxygen species are likely to play an important role in the maintenance of normal function of cells[4]. Due to this, an attempt was made to find out the radiation response of the enzymes involved in antioxidant function. Superoxide ($O^{\cdot}_2$) radicals formed in cells as an indirect consequence of irradiation could be selectively scavenged by SOD. Catalase protects the cell from H_2O_2, which is one of the detrimental to biological systems and maintains the concentration of O_2 either from repeated rounds of

chemical reduction or from the direct interaction with toxins[60]. The enhanced activity of DTD might protect the irradiated cells against free radical damage by means of its ability to generate and maintain the reduced antioxidant state of coenzyme Q (CoQ) in the membranes[61,62] as well as to act as two electron CoQ reductase[63]. GST might catalyse antioxidant processes of thiol compounds and protect cells from electrophiles, free radical-induced damage and oxidative stress[29,64,65].

Enhanced activities of antioxidant enzymes are expected to metabolize free radicals and their products in turn protect the cells against radiation effect. It is important that the activities of all enzymes studied were enhanced[66-69]. All these enzymes are known to function co-operatively. Our finding also supports this aspect. Hoechst 33258 also enhanced the specific activities of antioxidant enzymes (Table 4). This increase is expected to contribute in enhancement of antioxidant status of splenocytes. Therefore, apart from scavenging the free radicals, Hoechst 33258 seems to protect cells through modulation of antioxidant function of cell.

GSH is important member of the non-enzymatic antioxidant defense system. GSH has a redox potential of around (-) 230 mv, which makes it behave as an antioxidant and in turn efficient free radical scavenger. It was important that the level of GSH content of splenocytes following irradiation was found to be increased. The level of GSH was decreased in the presence of Hoechst 33258 and need to be evaluated further, but there was no significant change in unirradiated control group. Lowering of cellular GSH content also indicates generation of large quantity of ROS[70], in this way H33258 spares cellular GSH (both share the common property of scavenging $OH^{\cdot}$ radicals). It is important that Hoechst 33258 enhances and maintains the equilibrium of co-operative function of antioxidative enzymes. Possibly Hoechst 33258 is itself a free radical scavenger viz. this role is played by Hoechst-33258 (ref. 71).

Results of the present work suggested that Hoechst 33258 has an ability to protect the biological system against radiation induced apoptosis through inhibition of membrane related events as it reduces the lipid peroxidation, fluidity, $NO^{\cdot}$, GSH, fragmentation of DNA, DNA binding activity of NF-kB and AP-1. Hoechst 33258 also enhanced specific activities of antioxidative enzymes which play an important role in the maintenance of normal function of cells. It also appears that radiation protection has been closely linked with modulation of membrane related events and elevation of antioxidant status. Earlier, the work on Hoechst 33258 was mainly focussed on their binding property with DNA, ignoring biochemical pathways and membrane related events. Therefore, the present results suggest an alternative mechanism to explain the radioprotective potential of Hoechst 33258 to cells and tissues which are highly radiosensitive. Examples include lymphocytes, which are mature functional cells, have relatively high radiosensitivity in comparison with other leukocyte types. Radiation-induced apoptosis in lymphocytes causes the depletion of peripheral blood lymphocytes and leads to immunosuppression[72]. Therefore, it is proposed that Hoechst 33258 treatment prior to irradiation may have an even greater benefit in radiotherapy since Hoechst 33258 effectively prevented the radiation-induced apoptosis of peripheral blood lymphocytes.

Acknowledgement

Authors gratefully acknowledge the financial assistance of the Indian Council of Medical Research, New Delhi, India, in the form of Senior Research Fellowship to Zubaida Khatoon.

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